

PATENT

#14
DJO
8/29/80

DECLARATION UNDER 37 CFR 1.132

Sir/Madam:

(1) I am the sole inventor in the above-identified application.

(3) Objective

(4) Development

The following experimental studies were conducted by me, or under my supervision, to show that the method of the invention is broadly suitable for use with anti-sense oligos

designed as taught by this application and targeted to any and all adenosine receptor mRNAs.

Anti-sense Oligo I was disclosed in the above-identified patent application. For the present work, I designed 5 additional anti-sense phosphorothioate oligos, one oligo targeted to the adenosine A₁ receptor (Oligo II), one oligo targeted to the adenosine A_{2b} receptor (Oligo V), and two oligos targeted to the adenosine A₃ receptor (Oligos III and IV), and one anti-sense phosphodiester oligo (Oligo I-PD) having the same sequence as Oligo I, as described in the above-identified patent application. These anti-sense oligos were designed for therapy on a selected species as described in the above patent application and are generally specific for that species, unless the segment of the adenosine receptor mRNA of other species elected happen to have similar sequences. All anti-sense oligos were prepared as described below, and tested in vivo in a rabbit model for bronchoconstriction, inflammation and allergy, which have breathing difficulties and impeded lung airways, as is the case in ailments such as asthma, as described in the above-identified application.

(5) Methods

(a) Anti-sense DNA

Six oligos and their effects in a rabbit model were studied and the results of these studies are reported and discussed below. Five of these oligos were selected for this study to complement the data on SEQ ID NO: 1 (Oligo I), which is anti-sense to the adenosine A₁ receptor mRNA provided in the above-identified patent application. The six new oligos are identified as anti-sense Oligos I (SEQ ID NO: 1) and II (targeted to a different region of the adenosine A₁ receptor mRNA), Oligo V (targeting the adenosine A_{2b} receptor mRNA), and anti-sense Oligos III and IV (targeting two different regions of the adenosine A₃ receptor mRNA). The sixth oligo (Oligo I-PD) is a phosphodiester version of Oligo I (SEQ. ID NO:1). The design and synthesis of these anti-sense oligos was performed in accordance with the teachings of the above-identified patent application, particularly of Example 1.

(I) Anti-sense Oligo I: The above-identified application disclosed anti-sense oligonucleotide I to the human A₁ adenosine receptor mRNA (EPI 2010; SEQ. ID NO: 1).

Anti-sense oligo I is 21 nucleotide long, overlaps the initiation codon, and has the following sequence.

5'- GAT GGA GGG CGG CAT GGC GGG -3'

The oligo I was previously shown to abrogate the adenosine-induced bronchoconstriction in allergic rabbits, and to reduce allergen-induced airway obstruction and bronchial hyperresponsiveness (BHR). See, Nyce, J. W. & Metzger, W. J., Nature, 385:721 (1977), a copy of which is enclosed.

(II) Anti-sense Oligo II: A phosphorothioate anti-sense oligo (EPI 2014) was designed in accordance with the invention to target the rabbit adenosine A₁ receptor mRNA region +936 to +956 relative to the initiation codon (start site). The anti-sense oligo II is 21 nucleotide long, and has the following sequence.

5'-CTC GTC GCC GTC GCC GGC GGG-3'

(III) Anti-sense Oligo III: A phosphorothioate anti-sense oligo (EPI 2046) was designed in accordance with the invention to target the anti-sense A₃ receptor mRNA region +3 to + 22 relative to the initiation codon start site. The anti-sense oligo III is 20 nucleotide long, and has the following sequence.

GGG TGG TGC TAT TGT CGG GC-3'

(IV) Anti-sense Oligo IV: A phosphorothioate anti-sense oligo (EPI 2047) was designed in accordance with the invention to target the adenosine A₃ receptor mRNA region + 386 to + 401 relative to the initiation codon (start site). The anti-sense oligo IV is 15 nucleotide long, and has the following sequence.

5'-GGC CCA GGG CCA GCC-3'

(V) Anti-sense Oligo V: A phosphorothioate anti-sense oligo (EPI 2099) was designed in accordance with the invention to target the adenosine A_{2b} receptor mRNA region -21 to -1 relative to the initiation codon (start site). The anti-sense oligonucleotide V is 21 nucleotide long, and has the following sequence.

5'-GGC CGG GCC AGC CGG GCC CGG-3'

(VI) A₁ Mismatch Oligos: Two different mismatched oligonucleotides having the following sequences were used as controls for anti-sense oligo I (SEQ. ID NO: 1) described in (a) above.

A₁ MM 5'-GTA GGT GGC GGG CAA GGC GGG-3'
A₁ MM2 5'-GAT GGA GGC GGG CAT GGC GGG-3'

Anti-sense oligo I and the two mismatch anti-sense oligos had identical base content and general sequence structure. Homology searches in GENBANK (release 85.0) and EMBL (release 40.0) indicated that the anti-sense oligo I was specific, not only for the human, but also for the rabbit, adenosine A₁ receptor genes, and that the mismatched controls were not candidates for hybridization with any known human or animal gene sequence.

(VII) Anti-sense Oligo A₁-PD: A phosphodiester anti-sense oligo having the same nucleotide sequence as Oligo I was designed as disclosed in the above-identified application. Anti-sense oligo I-PD is 21 nucleotide long, overlaps the initiation codon, and has the following sequence.

5'- GAT GGA GGG CGG CAT GGC GGG -3'

(VIII) Controls: Each rabbit was administered 5.0 ml aerosolized sterile saline following the same schedule as for the anti-sense oligos in (II), (III), and (IV) above.

(b) Synthesis of Anti-sense Oligos

Phosphorothioate anti-sense oligos having the sequences described in (a) above, were synthesized on an Applied Biosystems Model 396 Oligonucleotide Synthesizer, and purified using NENSORB chromatography (DuPont, DE). TETD (tetraethylthiuram disulfide) was used as the sulfurizing agent during the synthesis. Anti-sense oligonucleotide II (EPI 2014), anti-sense oligonucleotide III (EPI 2046) and anti-sense oligonucleotide IV (EPI 2047) were each synthesized and purified in this manner.

(c) Preparation of Allergic Rabbits

Neonatal New Zealand white Pasturella-free rabbits were immunized intraperitoneally within 24 hours of birth with 0.5 ml of 312 antigen units/ml house dust mite (*D. farinae*) extract (Berkeley Biologicals, Berkeley, CA) mixed with 10% kaolin as previously described (Metzger, W. J. In: Late Phase Allergic Reactions, (Dorsch, W., Ed.) CRC Handbook, pp 347-362, CRC Press, Boca Raton, 1990; Ali, S., Metzger, W. J. and Mustafa, S. J., Am. J. Resp. Crit. Care Med. 149, 908, 1994). Immunizations were repeated weekly for the first month and then biweekly until the age of 4 months. These rabbits preferentially produce allergen-specific IgE antibody, typically respond to aeroallergen challenge with both an early and late-phase asthmatic response, and show

bronchial hyper responsiveness (BHR). Monthly intraperitoneal administration of allergen (312 units dust mite allergen, as above) continues to stimulate and maintain allergen-specific IgE antibody and BHR. At 4 months of age, sensitized rabbits were prepared for aerosol administration as described by Ali et al. (199_) (Ali, S., Metzger, W. J. and Mustafa, S. J., Am. J. Resp. Crit. Care Med. 149 (1994)).

(d) Dose-response Studies

(i) Experimental Setup

Aerosols of either adenosine (0-20 mg/ml), or anti-sense or one of two mismatch oligonucleotides (5 mg/ml) were separately prepared with an ultrasonic nebulizer (Model 646, DeVilbiss, Somerset, PA), which produced aerosol droplets, 80% of which were smaller than 5 μ m in diameter. Equal volumes of the aerosols were administered directly to the lungs *via* an intratracheal tube.

The animals were randomized, and administered aerosolized adenosine. Day 1 pre-treatment values for sensitivity to adenosine were calculated as the dose of adenosine causing a 50% loss of compliance (PC₅₀ Adenosine). The animals were then administered either the aerosolized anti-sense or one of the mismatch anti-sense oligos *via* the intratracheal tube (5 mg/1.0 ml), for 2 minutes, twice daily for 2 days (total dose, 20 mg). Post-treatment PC₅₀ values were recorded (post-treatment challenge) on the morning of the third day. The results of these studies are provided in (6)(a)(iii) below.

(ii) Crossover Experiments

For some experiments utilizing anti-sense oligo I (SEQ ID NO: 1) and a corresponding mismatch control oligonucleotide A1MM, following a 2 week interval, the animals were crossed over, with those previously administered the mismatch control A₁MM, now receiving the anti-sense oligo I, and those previously treated with the anti-sense oligo I, now receiving the mismatch control A₁MM oligo.

The number of animals per group was as follows. For mismatch A₁MM (control 1), n=7, since one animal was lost in the second control arm of the experiment due to technical difficulties, for mismatch A₁MM2 n=4 (control 2) and for A₁AS anti-sense oligo I, n=8.

The A₁MM2 oligo-treated animals were analyzed separately and were not part of the cross-over experiment. The treatment methods and measurements employed following the cross-over were identical to those employed in the first arm of the experiment.

In 6 of the 8 animals treated with the anti-sense oligo I (SEQ. ID NO: 1), no PC₅₀ value could be obtained for adenosine doses of up to 20 mg/ml, which is the limit of solubility of adenosine. Accordingly, the PC₅₀ values for these animals were assumed to be 20 mg/ml for calculation purposes. The values given, therefore, represent a minimum figure for the effectiveness of the anti-sense oligonucleotides of the invention. Other groups of allergic rabbits (n=4 for each group) were administered 0.5 or 0.05 mg doses of the anti-sense oligo I (A₁AS; SEQ ID NO: 1), or the A₁MM oligo in the manner and according to the schedule described above (the total doses being 2.0 or 0.2 mg). The results of these studies are provided in (6)(a)(iv) below.

(e) Anti-sense Oligo Formulation

Each one of anti-sense oligos were separately solubilized in an aqueous solution and administered as described for anti-sense oligo I in (e) above, in four 5 mg aliquots (20 mg total dose) by means of a nebulizer via endotracheal tube, as described above.

The results obtained for anti-sense oligo I and its mismatch controls confirmed that the mismatch controls are equivalent to saline, See, Table 1 of Nyce & Metzger, Nature 385, 721-725, 1997. Because of this finding, saline was used as a control for pulmonary function studies employing anti-sense oligos II, III and IV.

(f) Specificity of Oligo I
for Adenosine A₁ Receptor
(Receptor Binding Studies)

Tissue from airway smooth muscle was dissected to primary, secondary and tertiary bronchi from rabbits which had been administered 20 mg oligo I (A₁AS; SEQ ID NO: 1; EPI 2010) in 4 divided doses over a period of 48 hours as described above. A membrane fraction was prepared according to the method of Ali et al. See, Ali, S., et al., Am. J. Resp. Crit. Care Med. 149,: 908 (1994).

The protein content was determined by the method of Bradford and plasma membranes were incubated with 0.2 U/ml adenosine deaminase for 30 minutes at 37°C to remove endogenous adenosine. See, Bradford, M. M. Anal. Biochem. 72, 240-254 (1976). The binding of [^3H]DPCPX, [^3H]NPC17731, or [^3H]CGS-21680 was measured as described by Jarvis et al. See, Jarvis, M.F., et al., Pharmacol. Exptl. Ther. 251, 888-893 (1989). The results of this study are shown in Table 1 and discussed in (6)(a)(ii) below.

**(g) Pulmonary Function Measurements
(Compliance C_{DYN} and Resistance)**

At 4 months of age, the immunized animals were anesthetized and relaxed with 1.5 ml of a mixture of ketamine HCl (35 mg/kg) and acepromazine maleate (1.5 mg/kg) administered intramuscularly. After induction of anesthesia, allergic rabbits were comfortably positioned supine on a soft molded animal board. Salve was applied to the eyes to prevent drying, and they were closed. The animals were then intubated with a 4.0 mm intermediate high-low cuffed Murphy 1 endotracheal tube (Mallinckrodt, Glen Falls, NY), as previously described by Zavala and Rhodes. See, Zavala and Rhodes, Proc. Soc. Exp. Biol. Med. 144: 509-512 (1973). A polyethylene catheter of OD 2.4 mm (Becton Dickinson, Clay Adams, Parsippany NJ) with an attached thin-walled latex balloon was passed into the esophagus and maintained at the same distance (approximately 16 cm) from the mouth throughout the experiment. The endotracheal tube was attached to a heated Fleisch pneumotach (size 00; DEM Medical, Richmond, VA), and the flow (v) measured using a Validyne differential pressure transducer (Model DP-45-16-1927, Validyne Engineering, Northridge, CA), driven by a Gould carrier amplifier (Model 11-4113, Gould Electronics, Cleveland, OH).

An esophageal balloon was attached to one side of the Validyne differential pressure transducer, and the other side was attached to the outflow of the endotracheal tube to obtain transpulmonary pressure (P_{tp}). The flow was integrated to yield a continuous tidal volume, and the measurements of total lung resistance (R_{t}) and dynamic compliance (C_{dyn}) were made at isovolumetric and zero flow points. The flow, volume and pressure were recorded on an eight channel Gould 2000 W high-frequency recorder and C_{dyn} was calculated using the total volume and the difference in P_{tp} at zero flow, and R_{t} was calculated as the ratio of P_{tp} and V at midtidal lung volumes. These calculations were made automatically with the Buxco automated pulmonary mechanics respiratory analyzer (Model 6, Buxco Electronics, Sharon,

CT), as previously described by Giles et al. See, Giles et al., Arch. Int. Pharmacodyn. Ther. 194: 213-232 (1971). The results obtained upon administration of oligo II on allergic rabbits are shown and discussed in (6)(b) below.

(h) Measurement of Bronchial Hyperresponsiveness (BHR)

Each allergic rabbit was administered histamine by aerosol to determine their baseline hyperresponsiveness. Aerosols of either saline or histamine were generated using a DeVilbiss nebulizer (DeVilbiss, Somerset, PA) for 30 seconds and then for 2 minutes at each dose employed. The ultrasonic nebulizer produced aerosol droplets of which 80% were <5 micron in diameter. The histamine aerosol was administered in increasing concentrations (0.156 to 80 mg/ml) and measurements of pulmonary function were made after each dose. The B4R was then determined by calculating the concentration of histamine (mg/ml) required to reduce the C_{dyn} 50% from baseline ($PC_{50 \text{ Histamine}}$).

(i) Cardiovascular Effect of Anti-sense Oligo I

The measurement of cardiac output and other cardiovascular parameters using Cardiomax™ utilizes the principal of thermal dilution in which the change in temperature of the blood exiting the heart after a venous injection of a known volume of cool saline is monitored. A single rapid injection of cool saline was made into the right atrium via cannulation of the right jugular vein, and the corresponding changes in temperature of the mixed injectate and blood in the aortic arch were recorded via cannulation of the carotid artery by a temperature-sensing miniprobe.

Twelve hours after the allergic rabbits had been treated with aerosols of oligo I (EPI 2010; SEQ. ID NO: 1) as described in (d) above, the animals were anesthetized with 0.3 ml/kg of 80% Ketamine and 20% Xylazine. This time point coincides with previous data showing efficacy for SEQ. ID NO: 1. See, Nyce & Metzger, (1997). A thermocouple was then inserted into the left carotid artery of each rabbit, and was then advanced 6.5 cm and secured with a silk ligature. The right jugular vein was then cannulated and a length of polyethylene tubing was inserted and secured.

A thermodilution curve was then established on a Cardiomax™ II (Columbus Instruments, Ohio) by injecting sterile saline at 20°C to determine the correctness of positioning of the thermocouple probe. After establishing the correctness of the position of the thermocouple, the femoral artery and vein were isolated. The femoral vein was used as a portal for drug injections, and the femoral artery for blood pressure and heart rate measurements. Once constant baseline cardiovascular parameters were established, Cardiomax™ measurements of blood pressure, heart rate, cardiac output, total peripheral resistance, and cardiac contractility were made.

(j) **Duration of Action of Oligo I**
(SEQ. ID NO: 1; EPI 2010)

Eight allergic rabbits received initially increasing log doses of adenosine by means of a nebulizer via an intra-tracheal tube as described in (f) above, beginning with 0.156 mg/ml until compliance was reduced by 50% (PC_{50} Adenosine) to establish a baseline. Six of the rabbits then received four 5 mg aerosolized doses of (SEQ. ID NO: 1; EPI 2010) as described above. Two rabbits received equivalent amounts of saline vehicle as controls. Beginning 18 hours after the last treatment, the PC_{50} Adenosine values were tested again. After this point, the measurements were continued for all animals each day, for up to 10 days. The results of this study are shown in Figures 5 and 6 and discussed in (6)(a)(vii) below.

(k) **Reduction of Adenosine A_{2b} Receptor**
Number by Anti-sense Oligo V

Sprague Dawley rats were administered 2.0 mg respirable anti-sense oligo V (EPI 2099) three times over two days using an inhalation chamber as described above. Twelve hours after the last administration, lung parenchymal tissue was dissected and assayed for adenosine A_{2b} receptor binding using [311]-NECA as described by Nyce & Metzger (1997). Controls were conducted by administration of equal volumes of saline. The results are significant at $p < 0.05$ using Student's paired t test, and are shown in Figure 9 and discussed in section (6) (c) below.

(l) **Comparison of Oligo I & Corresponding**
Phosphodiester Oligo VI (EPI 2010-PD)

Oligo I (EPI 2010) countered the effects of adenosine and eliminated sensitivity to it for adenosine amount up to 20 mg adenosine/5.0 ml (the limit of solubility of adenosine). Oligo VI (EPI 2010-PD), the phosphodiester version of the oligonucleotide sequence, was completely ineffective when tested in the same manner. Both compounds have identical sequence, differing only in the presence of phosphorothioate residues in Oligo I (EPI 2010)., and were delivered as an aerosol as described above and in Nyce & Metzger (1997). Significantly different at $p < 0.001$, Student's paired t test. The results are discussed in section (6) (d) and in Figure 10.

(6) Results

(a) Anti-sense Oligo I

(i) Prior Work

The nucleotide sequence and other data for anti-sense oligo I (SEQ. ID NO: 1), which is specific for the adenosine A_1 receptor, was provided in the original application. In addition, the application also contained experimental data showing the effectiveness of oligo I in down regulating the receptor number and activity.

Further information on anti-sense oligo I was provided in a publication by my group. See, Nyce, J. W., and Metzger, W. J., Nature 385:721 (1997) (copy enclosed). The Nyce & Metzger (1997) publication provided data showing that the anti-sense oligo I (SEQ. ID NO: 1):

- (1) Reduces the number of adenosine A_1 receptors in the bronchial smooth muscle of allergic rabbits in a dose-dependent manner. See, Table 1 of Nyce & Metzger (1997).
- (2) Attenuates adenosine-induced bronchoconstriction and allergen-induced bronchoconstriction. See, Figure 4 of Nyce & Metzger (1997).
- (3) Attenuates bronchial hyperresponsiveness as measured by PC_{50} histamine, a standard measurement to assess bronchial hyperresponsiveness. This result clearly demonstrates anti-inflammatory activity of the anti-sense oligo I. See, Figure 4 of Nyce & Metzger (1997).
- (4) As expected, because it was designed to target it, is totally specific for the adenosine A_1 receptor, and has no effect at all at any dose on either the very closely related adenosine A_2 receptor or the related bradykinin B_2 receptor. See, Table 1

of Nyce & Metzger (1997), and Figure 2 accompanying this Declaration.

- (5) Mismatch control molecules (MM1 and MM2; See, Figure 1 of Nyce & Metzger) had identical base composition and molecular weight but differed from the anti-sense oligo I (SEQ ID NO: 1) by 6 and 2 mismatches, respectively. These mismatches, which are the minimum possible while still retaining identical base composition, produced absolutely no effect upon any of the targeted receptors (A_1 , A_2 or B_2). See, Figure 1 of Nyce & Metzger (1997).

These results, along with a complete lack of prior art on the use of anti-sense oligonucleotides, such as oligo I, targeted to the adenosine A_1 receptor, show the unexpected results obtain by me. More generally, the anti-sense oligonucleotides of the invention which are directed to adenosine receptor lung targets, particularly targets associated with asthma, are not only unobvious over the art at large, but have been broadly enabled by the prior work reported in the above-identified application, the work reported in Nyce & Metzger (1997), and the further work reported here. These collective showings clearly enable and show the effectiveness, for their intended use, of the claimed agent and method for reducing or treating bronchoconstriction and lung inflammation.

**(ii) Oligo I Significantly Reduces
Response to Adenosine Challenge**

The receptor binding experiment is described in (5)(f), and the results shown in Figures 1 and 2 accompanying this Declaration, and in Table 1 below which shows the binding characteristics of the adenosine A_1 -selective ligand [3H]DPCPX and the bradykinin B_2 -selective ligand [3H]NPC 17731 in membranes isolated from airway smooth muscle of A_1 adenosine receptor and B_2 bradykinin receptor anti-sense- and mismatch-treated allergic rabbits.

Table 1: Binding Characteristics of Three Anti-Sense Oligos

Treatment ¹	A ₁ receptor		B ₂ receptor	
	K _d	B _{max}	K _d	B _{max}
A₁AS				
20 mg	0.36±0.029 nM	19±1.52 fmoles*	0.39±0.031 nM	14.8±0.99 fmoles
2 mg	0.38±0.030 nM	32±2.56 fmoles*	0.41±0.028 nM	15.5±1.08 fmoles
0.2 mg	0.37±0.030 nM	49±3.43 fmoles	0.34±0.024 nM	15.0±1.06 fmoles
A₁MM (Control)				
20 mg	0.34±0.027 nM	52.0±3.64	0.35±0.024 nM	14.0±1.0 fmoles
2 mg	0.37±0.033 nM	51.8±3.88	0.38±0.028 nM	14.6±1.02 fmoles
B₂A (Bradykinin Receptor)				
20 mg	0.36±0.028 nM	45.0±3.15	0.38±0.027 nM	8.7±0.62 fmoles*
2 mg	0.39±0.035 nM	44.3±2.90	0.34±0.024 nM	11.9±0.76 fmoles**
0.2 mg	0.40±0.028 nM	47.0±3.76	0.35±0.028 nM	15.1±1.05 fmoles
B₂MM (Control)				
20 mg	0.39±0.031 nM	42.0±2.94	0.41±0.029 nM	14.0±0.98 fmoles
2 mg	0.41±0.035 nM	40.0±3.20	0.37±0.030 nM	14.8±0.99 fmoles
0.2 mg	0.37±0.029 nM	43.0±3.14	0.36±0.025 nM	15.1±1.35 fmoles
Saline Control	0.37±0.041	46.0±5.21	0.39±0.047	14.2±1.35

¹ Refers to total oligo administered in four equivalently divided doses over a 48 hour period. Treatments and analyses were performed as described in methods. Significance was determined by repeated-measures analysis of variance (ANOVA), and Tukey's protected t test. n = 4-6 for all groups.

* Significantly different from mismatch control- and saline-treated groups, p<0.001;

**Significantly different from mismatch control- and saline-treated groups, p<0.05.

(iii) Dose-response Effect of Oligo I

Anti-sense oligo I (A₁AS; SEQ ID NO: 1) was found to reduce the effect of adenosine administration to the animal in a dose-dependent manner over the dose range tested as shown in Table 2 below and in Figure 2.

Table 2: Dose-Response Effect to Anti-sense Oligo I

Total Dose (mg)	PC ₅₀ Adenosine (mg Adenosine)
Anti-sense Oligo I	
0.2	8.32±7.2
2.0	14.0±7.2
20	19.5±0.34
A₁MM oligo (control)	
0.2	2.51±0.46
2.0	3.13± 0.71
20	3.25± 0.34

The above results were studied with the Student's paired t test and found to be statistically different, p=0.05

Figure 2 shows that oligo I, an anti-adenosine A₁ receptor oligo, acts specifically on the adenosine A₁ receptor, but not on the adenosine A₂ receptor. These results stem from the treatment of rabbits with anti-sense oligo I or mismatch control oligo as described in (5)(d)(i) above and in Nyce & Metzger (1997) (four doses of 5 mg spaced 8 to 12 hours apart via nebulizer via endotracheal tube), bronchial smooth muscle tissue excised and the number of adenosine A₁ and adenosine A₂ receptors determined as reported in Nyce & Metzger (1997).

**(iv) Specificity of Oligo I
for Target Gene Product**

Oligo I is specific for the adenosine A₁ receptor whereas its mismatch controls had no activity. Figure 1 depicts the results obtained from the cross-over experiment described in (5)(d)(ii) above and in Nyce & Metzger (1997). As may be seen from the top and lower panels of Figure 1, the two mismatch controls evidence no effect on the PC₅₀ Adenosine value. On the contrary, the administration of anti-sense oligo I (SEQ. ID NO: 1; EPI 2010) shows a seven-fold increase in the PC₅₀ Adenosine value. The results shown in Figure 1 above clearly indicate that anti-sense oligo I (SEQ. ID NO: 1; EPI 2010) reduces the response (attenuates the sensitivity) to exogenously administered adenosine when compared with a saline control. The results provided in Table 2 above clearly establish that the effect of the anti-sense oligo I is dose dependent (see, column 3 of Table 1).

Oligo I was also shown to be totally specific for the adenosine A₁ receptor, (see, top 3 rows), inducing no activity at either the closely related adenosine A₂ receptor (see, Figure 2, right hand panel), or the bradykinin B₂ receptor (see, lines 8-10 of Table 2 above).

In addition, the results shown in Table 2 and Figure 2 establish that anti-sense oligo I decreases sensitivity to adenosine in a dose dependent manner, and that it does this in an anti-sense-dependent manner since neither of two mismatch control oligonucleotides show any effect on PC_{50 Adenosine} values or on the number of attenuation of adenosine A₁ receptors.

(v) **Effect on Aeroallergen-induced
Bronchoconstriction & Inflammation**

Oligo I was shown to significantly reduce the histamine-induced effect in the rabbit model when compared to the mismatch oligos. Figure 3 shows the effect of anti-sense Oligo I and the mismatch oligos on allergen-induced airway obstruction and bronchial hyperresponsiveness in allergic rabbits. Panels (a), (b), (c) and (d) represent the following.

Panel (a) shows the effect of anti-sense oligo I (A₁AS; SEQ. ID NO:1) on allergen-induced airway obstruction. As calculated from the area under the curve, the anti-sense oligo I significantly inhibited allergen-induced airway obstruction (55%, $p < 0.05$; repeated measures ANOVA, and Tukey's t test). Compare with panel (b) for mismatch A₁MM oligo (control).

Panel (b) shows the lack of effect of the mismatch oligo A₁MM (Control) on allergen induced airway obstruction.

Panel (c) shows the effect of the anti-sense oligo I (A₁AS; SEQ. ID NO:1) on allergen-induced BHR. As calculated from the PC_{50 Histamine} value, (A₁AS), the anti-sense oligo I significantly inhibited allergen-induced BHR in allergic rabbits (61%, $p < 0.05$; repeated measures ANOVA, Tukey's t test). Compare with Panel (d) for mismatch A₁MM oligo (Control).

Panel (d) shows a lack of effect of the A₁MM mismatch control on allergen-induced BHR.

The results shown in Figure 3, panel (a), indicate that anti-sense oligo I (SEQ. ID NO: 1; EPI 2010) is effective to protect against aeroallergen-induced bronchoconstriction (house dust mite). In addition, anti-sense oligo I was also found to be a potent inhibitor of dust mite-induced bronchial hyper responsiveness, as shown by its effects upon histamine sensitivity (panel(c)), indicating anti inflammatory activity for anti-sense oligo I.

(vi) **Anti-sense Oligo I is Free of Deleterious Side Effects**

Oligo I was shown to be free of side effects that might be toxic to the recipient. No changes in arterial blood pressure, cardiac output, stroke volume, heart rate,, total peripheral resistance or heart contractility (dPdT) were observed following administration of 2.0 or 20 mg oligo I. Figure 4 shows the results of the measurement of cardiac output (CO), stroke volume (SV), mean arterial pressure (MAP), heart rate (HR), total peripheral resistance (TPR), and contractility (dPdT) with a Cardiomax™ apparatus (Columbus Instruments, Ohio).

These results evidence that oligo I has no detrimental effect upon critical cardiovascular parameters. More particularly, this oligo does not cause hypotension. This finding is of particular importance because other phosphorothioate anti-sense oligonucleotides have been shown in the past to induce hypotension in some model systems. Furthermore, the adenosine A₁ receptor plays an important role in sinoatrial conduction within the heart. Attenuation of the adenosine A₁ receptor by anti-sense oligo I might be expected to result, therefore, in deleterious extrapulmonary activity in response to the downregulation of the receptor. This is not the case. The anti-sense oligo I does not produce any deleterious intrapulmonary effects and renders the administration of the low doses of the present anti-sense oligo free of unexpected, undesirable side effects.

This demonstrates that when oligo I is administered directly to the lung, it does not reach the heart in significant quantities to cause deleterious effects. This is in contrast to traditional adenosine receptor antagonists like theophylline which do escape the lung and can cause deleterious, even life-threatening effects outside the lung.

(vii) **Long Lasting Effect of Oligo I**

Oligo I evidenced a long lasting effect as evidenced by the PC₅₀ and Resistance values obtained upon its administration prior to adenosine challenge. Figures 5 and 6 show the values obtained.

Figure 5 shows the duration of the effect, with respect to the PC₅₀ of adenosine, of anti-sense oligo I when administered in four equal doses of 5 mg each by means of a nebulizer via an endotracheal tube, as described above. The effect of the agent is significant over days 1 to 8 after administration. When the effect of the anti-sense oligo I had disappeared, the animals were administered saline aerosols (controls), and the PC₅₀ Adenosine

values for all animals were measured again. Saline-treated animals showed base line PC_{50} adenosine values ($n=6$).

Figure 6 shows the duration of the effect (with respect to Resistance) for six allergic rabbits which were administered 20 mg of anti-sense oligo I (SEQ. ID NO: 1) as described above, upon airway resistance measured as also described above. The mean calculated duration of effect was 8.3 days for both PC_{50} adenosine ($p < 0.05$) and resistance ($p < 0.05$). These results show that anti-sense oligo I has an extremely long duration of action, which is completely unexpected.

(b) Anti-sense Oligo II

Anti-sense oligo II, targeted to a different region of the adenosine A_1 receptor mRNA, was found to be highly active against the adenosine A_1 -mediated effects. The results of the experiment are shown in Figure 7, which evidences the effect of anti-sense oligo II (EPI 2014) upon compliance (top figure) and resistance (lower figure) values when 20 mg anti-sense oligo II were administered to allergic rabbits as described above, and compliance and resistance values measured following an administration of adenosine as described above in (5)(g). Significant at $p < 0.05$ using paired t test, compliance; $p < 0.01$ for resistance.

The results of Figure 7 show that anti-sense oligo II, which targets the adenosine A_1 receptor, effectively maintains compliance and reduces resistance upon adenosine challenge.

(c) Antisense Oligos III and IV

Oligos III and IV were shown to be in fact specifically targeted to the adenosine A_2 receptor by their effect on reducing inflammation and the number of inflammatory cells present upon separate administration of 20 mg of the anti-sense oligos III and IV to allergic rabbits as described above. The number of inflammatory cells was determined in their bronchial lavage fluid 3 hours later by counting at least 100 viable cells per lavage. The data are provided in Figure 8.

Figure 8 shows the effect of anti-sense oligos III (EPI 2046) and IV (EPI 2047) upon granulocytes (top figure), and upon total cells in bronchial lavage (lower figure), following exposure to dust mite allergen. The results of Figure 8 show that anti-sense oligo IV and anti-sense oligo III are very potent anti-inflammatory agents in the asthmatic lung following exposure to dust mite allergen. As is known in the art, granulocytes, especially eosinophils, are the primary inflammatory cells of asthma, and the administration of anti-sense oligos III and IV reduced their numbers by 40% and 66%, respectively. Furthermore, anti-sense oligos IV and III also reduced the total number of cells in the bronchial lavage fluid by 40%

and 80%, respectively. This is also an important indicator of anti-inflammatory activity by the present anti-adenosine A_3 agents of the invention. Inflammation is known to underlie bronchial hyperresponsiveness and allergen-induced bronchoconstriction in asthma. Both anti-sense oligonucleotides III and IV, which are targeted to the adenosine A_3 receptor, are representative of an important new class of anti-inflammatory agents which may be designed to specifically target the lung receptors of each species.

(c) **Antisense Oligo V**

The anti-sense oligo V, targeted to the adenosine A_{2b} adenosine receptor mRNA was shown to be effective at countering adenosine A_{2b} -mediated effects, and at reducing the number of adenosine A_{2b} receptors present as shown in Figure 9.

(d) **Unexpected Superiority of Substituted over Phosphodiester-residue Oligo I-DS**

When oligos I and I-DS were separately administered to allergic rabbits as described above, and the rabbits were then challenged with adenosine, the phosphodiester oligo I-DS was ineffective in countering the effect of adenosine whereas oligo I showed high effectiveness. The results are shown in Figure 10.

(7) **Conclusions**

The work described and results discussed above indicate that all five non-phosphodiester anti-sense oligonucleotides designed in accordance with the teachings of the above-identified application were found to be highly effective at countering or reducing effects mediated by the receptors they are targeted to. That is, each and all of the two anti-sense oligos targeting an adenosine A_1 receptor mRNA, one targeting an adenosine A_{2b} receptor mRNA, and two targeting an A_3 receptor mRNA were shown capable of countering the effect of exogenously administered adenosine which is mediated by the specific receptor they are targeted to.

In addition, the results presented also show that the administration of the present agents results in extremely low or non-existent deleterious side effects or toxicity.

This represents 100% success in providing agents that are highly effective and specific in the treatment of bronchoconstriction and/or inflammation. This invention is applicable in the same manner to all adenosine receptor mRNAs.

Moreover, a comparison of the phosphodiester and a substituted version of the same oligonucleotide evidenced an unexpected superiority for the phosphothiorate oligonucleotide over the phosphodiester oligo.

These are clearly superior results which could not have been expected based on the knowledge of the art at the time of this invention. The experimental data and results provided are clearly enabling of a new class of agents comprising non-phosphodiester anti-sense oligonucleotides targeted to lung adenosine receptors.

(8) I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing thereon.

(9) Declarant further says not.

11/28/97
Date

Jonathan W. Nyce
Jonathan W. Nyce, Ph. D.

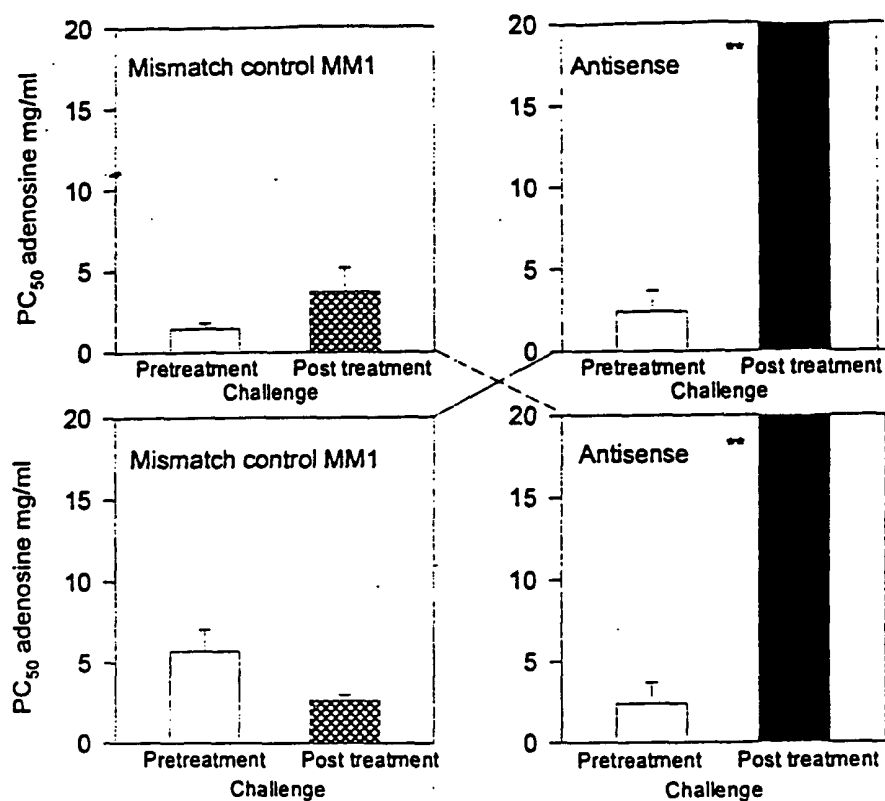


Figure 1: EPI 2010 Data Summary from both arms of crossover experiment

A ₁ MM Control		PC ₅₀ Adenosine A ₁ MM 2 Control		A ₁ AS	
Pre ODN	Post ODN	Pre ODN	Post ODN	Pre ODN	Post ODN
3.56 ± 1.02	3.25 ± 0.34	2.46 ± 0.50	2.81 ± 0.70	2.36 ± 0.68	>19.5 ± 0.34**

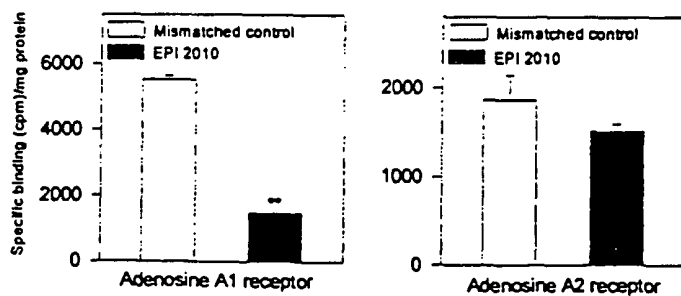


Figure 2.

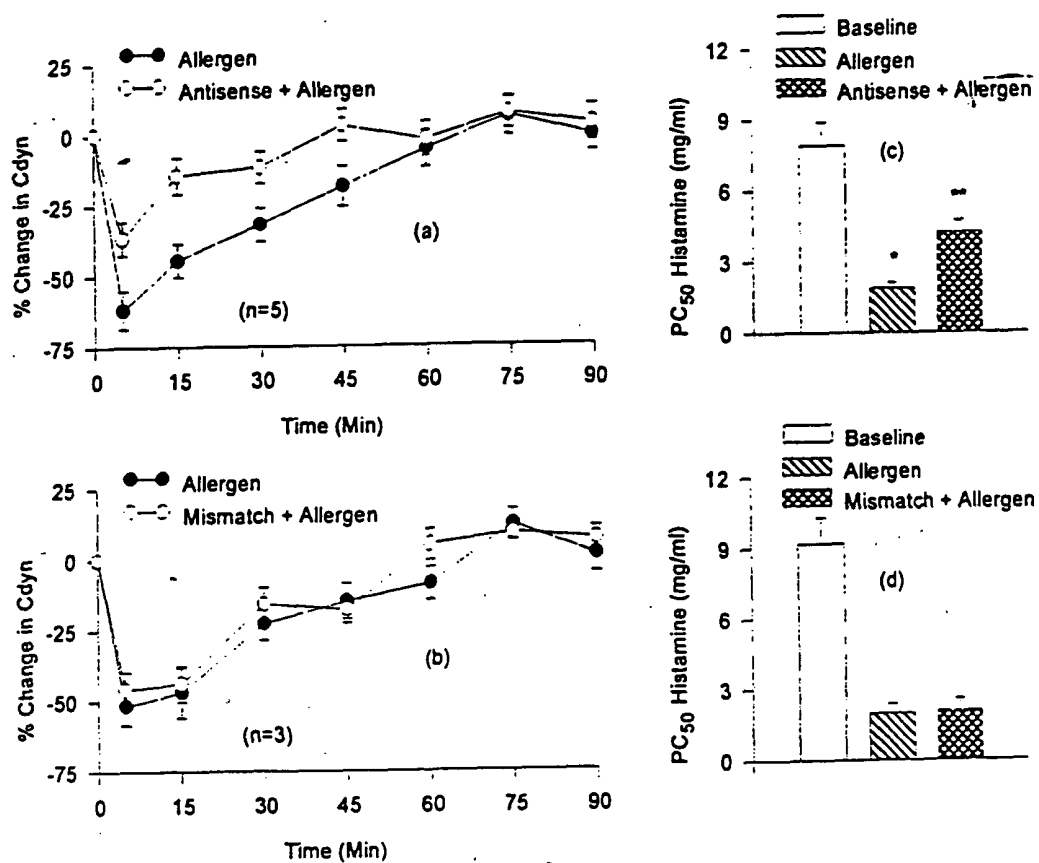


Figure 3.

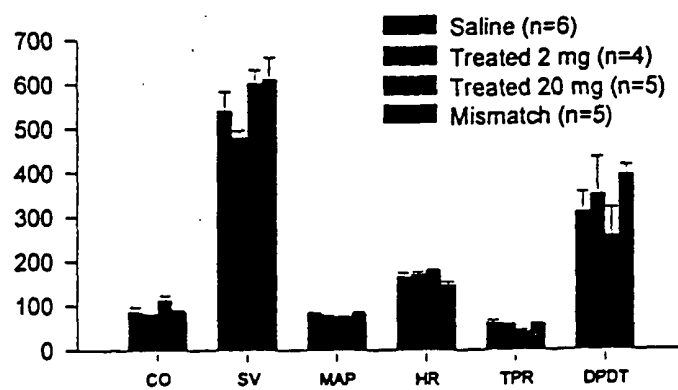


Figure 4.

EPI 2010 Duration of Effect (n=6)

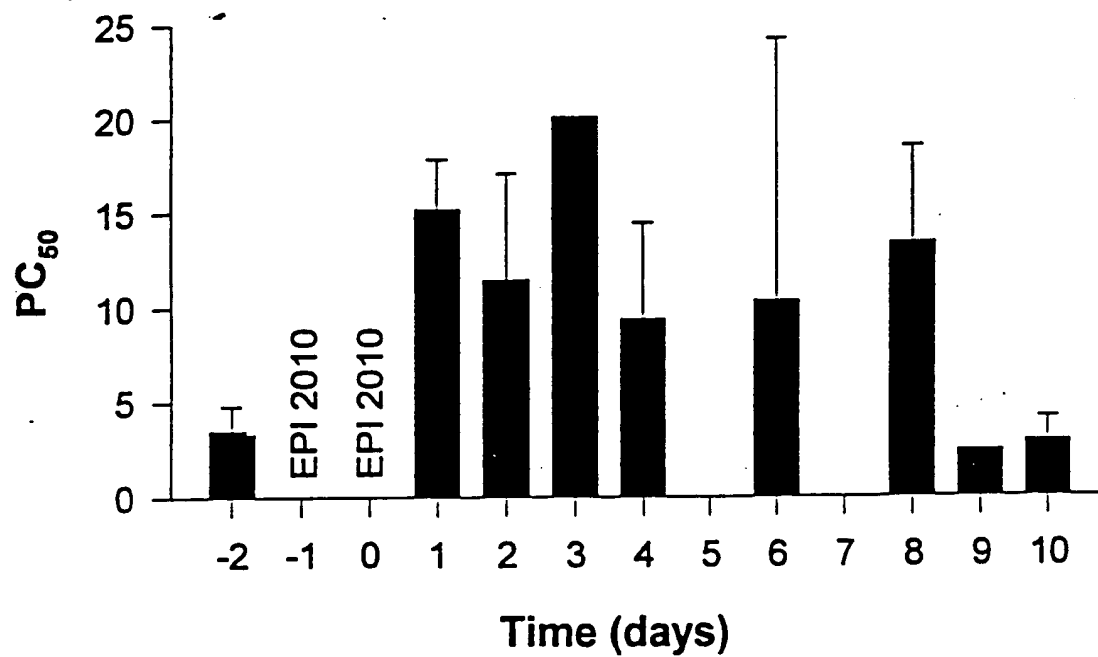
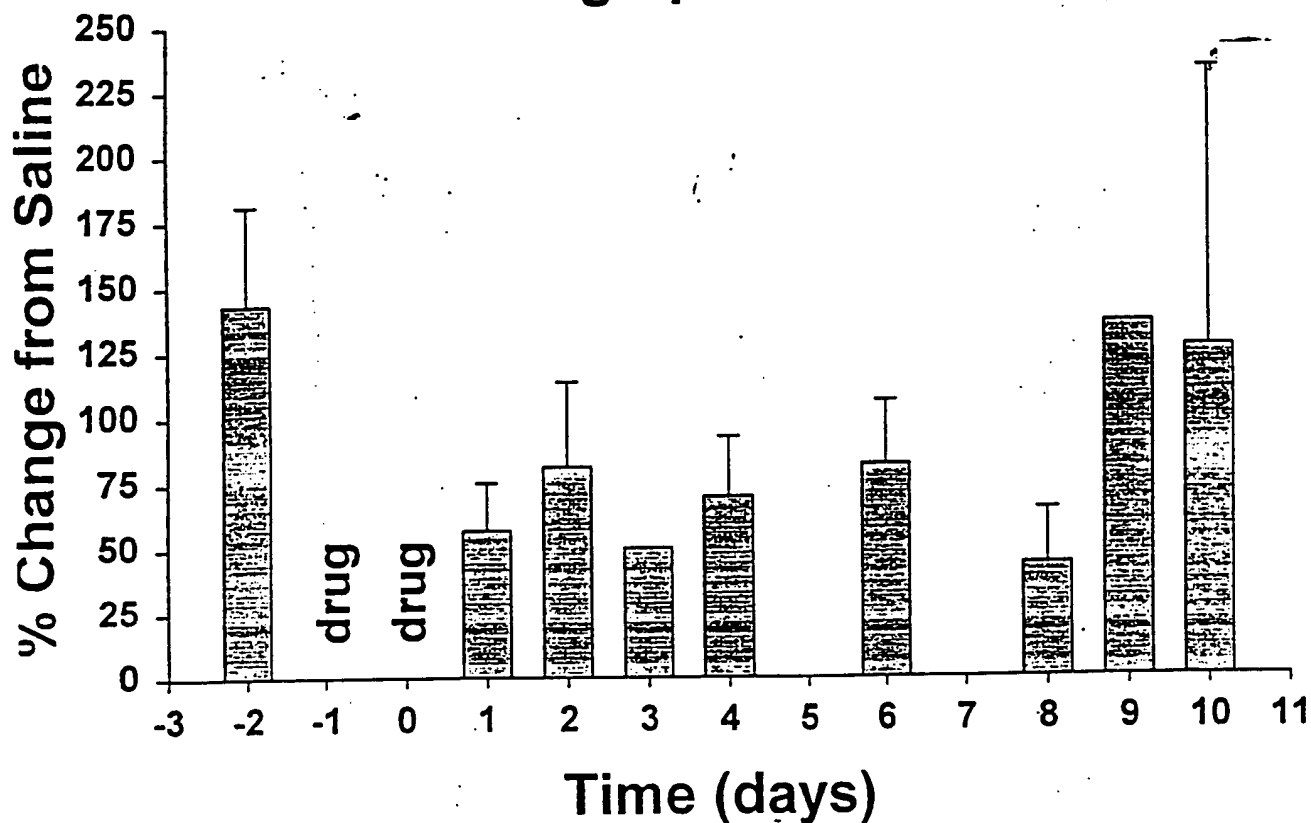


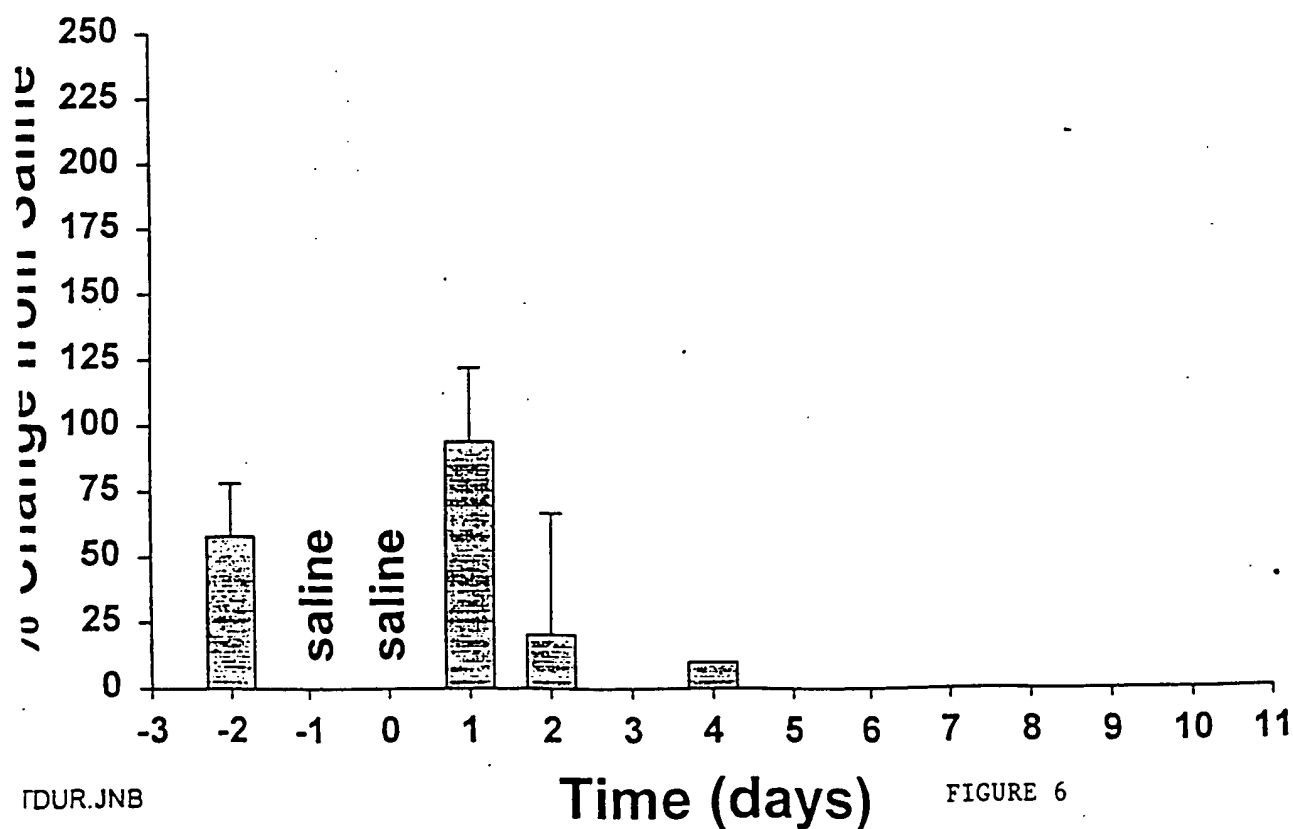
Figure 5.

EpiGenesis Duration Study Resistance Changes

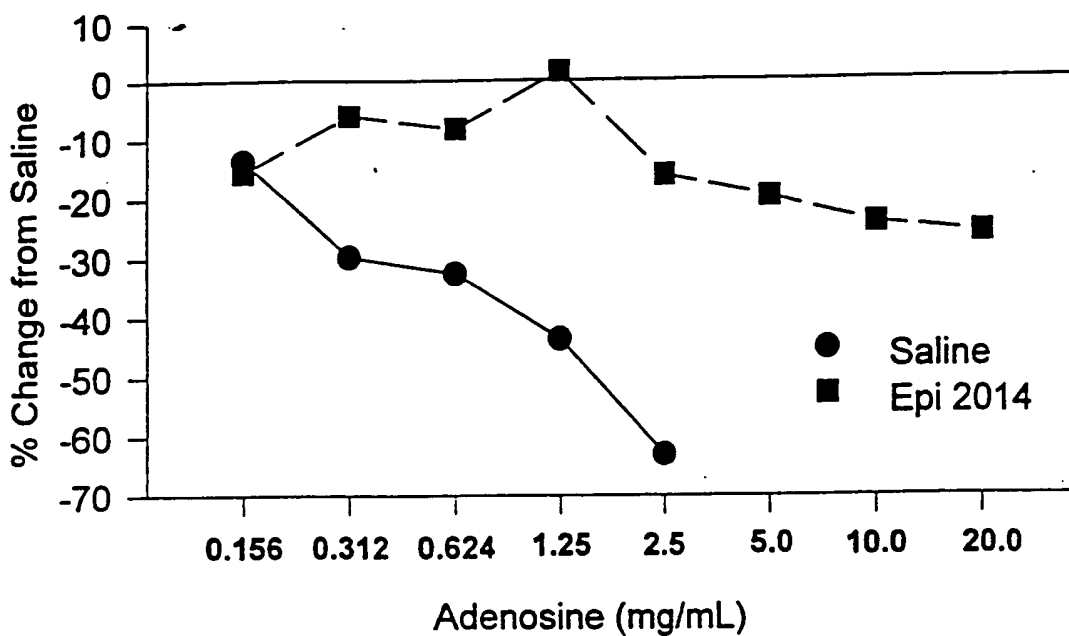
20 mg Epi2010 (n=6)



Saline (n=6)



Compliance, Epi 2014



Resistance, Epi 2014

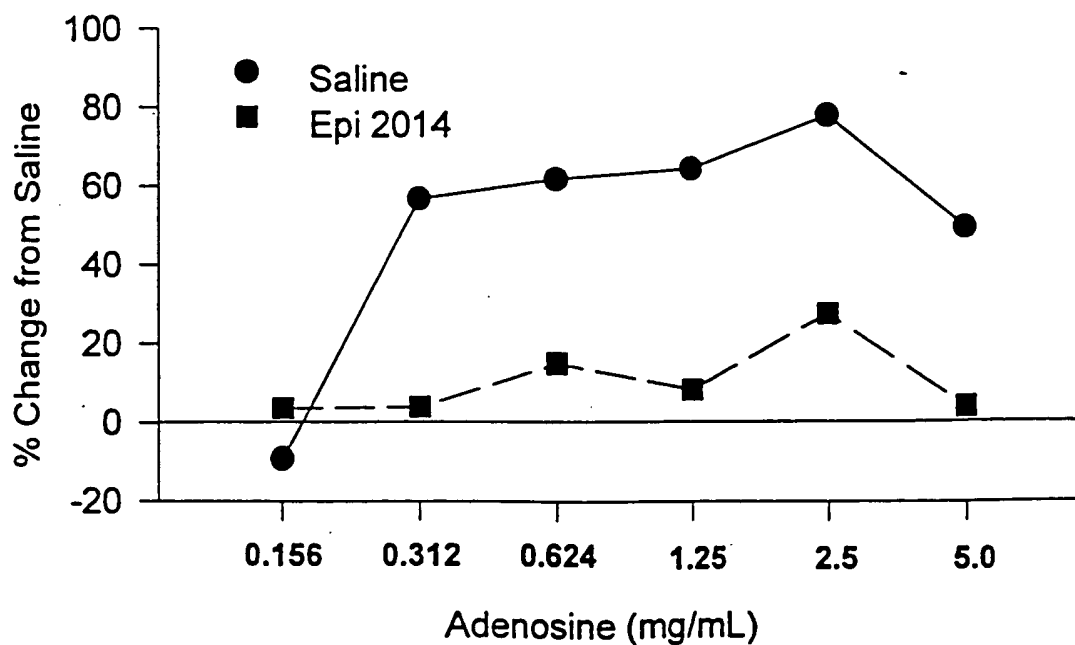


FIGURE 7

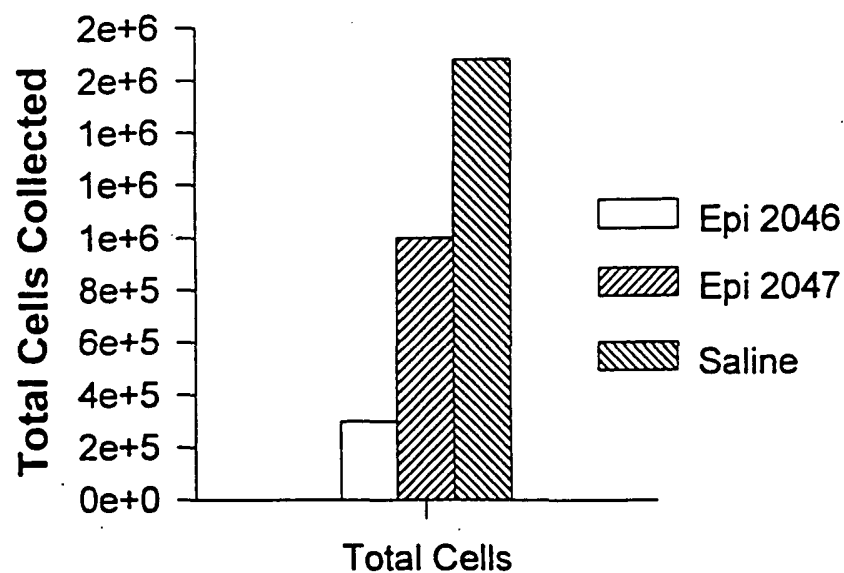
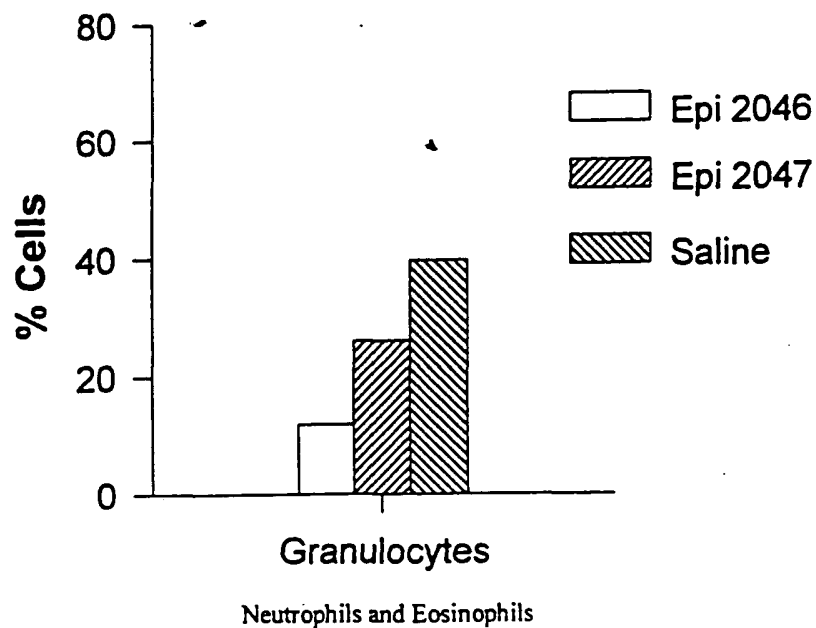


Figure 8. EPI 2046 and EPI 2047 effects upon granulocytes (upper panel) and total cells in bronchial lavage (lower panel) following exposure to dust mite allergen. Allergic rabbits were administered 20 mg EPI 2046 or 2047 as above, and inflammatory cells were enumerated in the bronchial lavage fluid 3 hours later. At least 100 viable cells per lavage were counted.

A2B Antisense in the rat

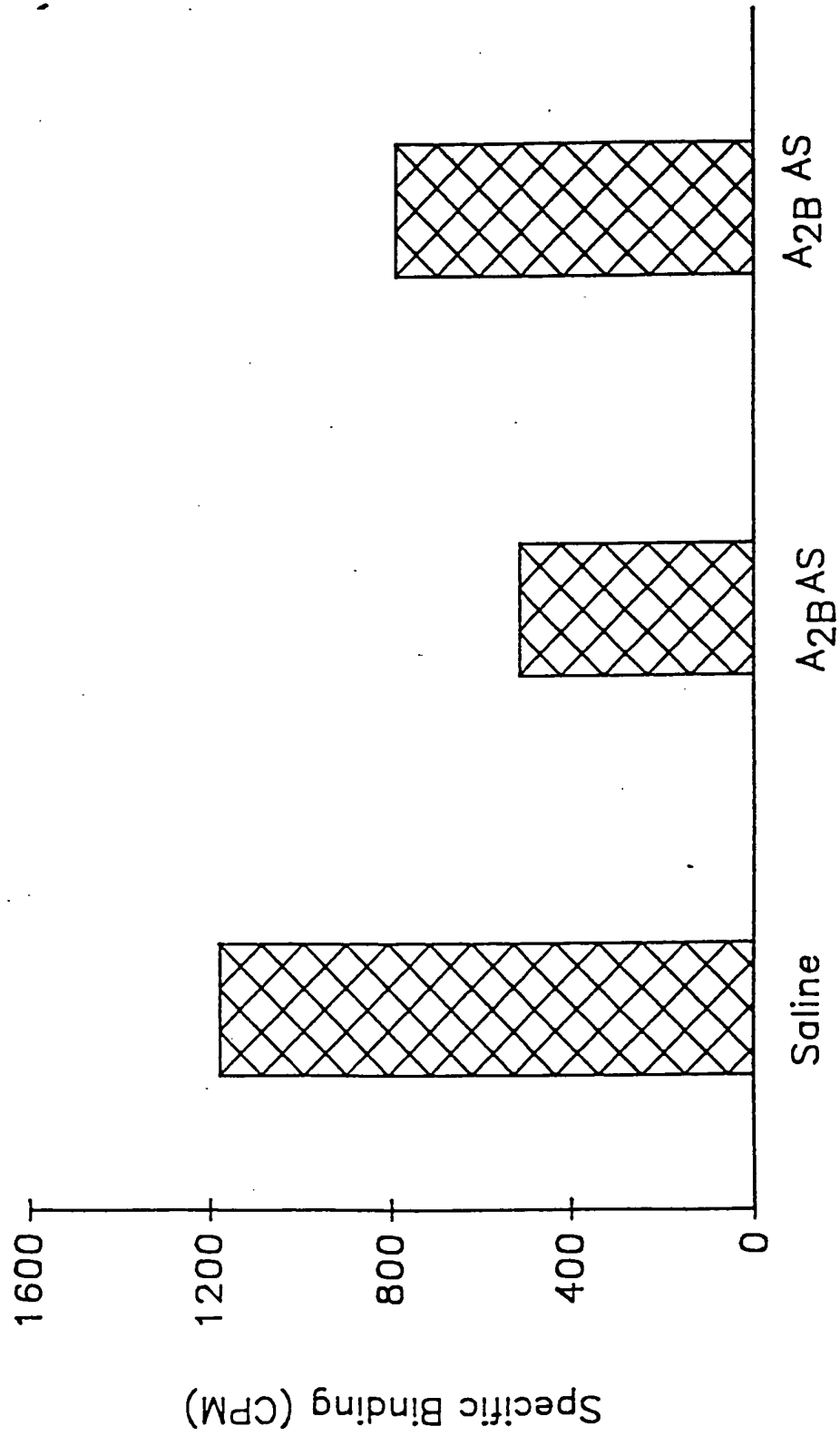


FIGURE 9

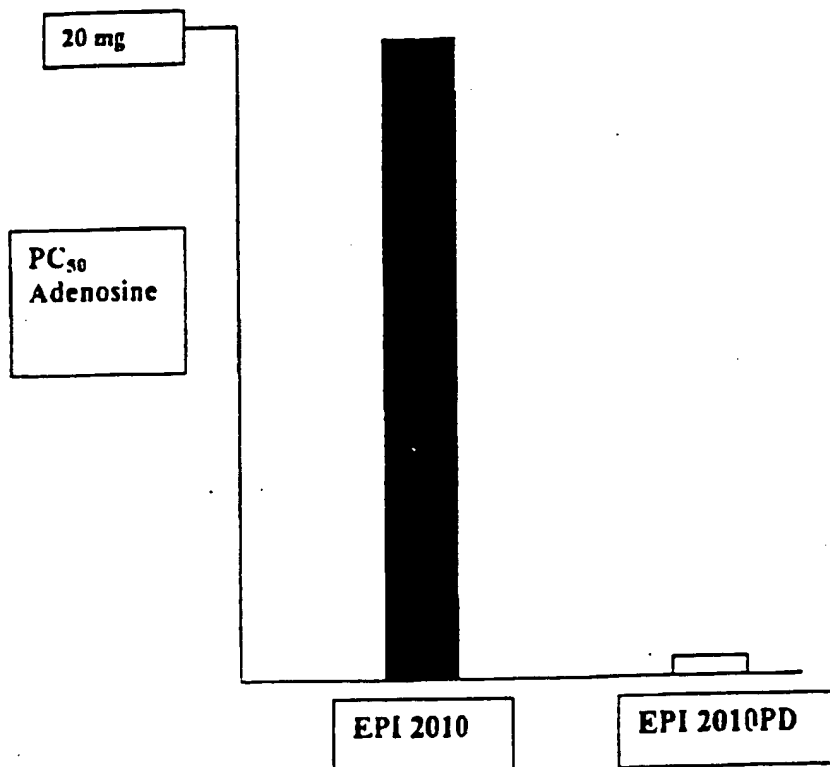


FIGURE 10